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Metformin Activates AMP-activated Protein Kinase by Promoting Formation of the $\alpha\beta\gamma$ Heterotrimeric Complex*

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Shumei Meng[‡1], **Jia Cao**[‡1], **Qiyi He**[‡], **Lishou Xiong**[‡], **Evan Chang**[‡], **Sally Radovick**[§], **Fredric E. Wondisford**[‡], **and Ling He**[‡2]

From the Divisions of [‡]*Metabolism and* [§]*Endocrinology, Departments of Pediatrics, Physiology, and Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287*

Background: The mechanism underlying the activation of AMPK by metformin remains unclear.
Results: Metformin promotes the formation of AMPK $\alpha\beta\gamma$ heterotrimeric complex.
Conclusion: The formation of the AMPK $\alpha\beta\gamma$ heterotrimeric complex augments AMPKα phosphorylation by LKB1 and prevents dephosphorylation by protein phosphatase.
Significance: Metformin-mediated formation of the AMPK $\alpha\beta\gamma$ heterotrimeric complex results in AMPK activation by elevating AMPK phosphorylation at Thr-172.

Metformin is the most widely prescribed oral anti-diabetic agent. Recently, we have shown that low metformin concentrations found in the portal vein suppress glucose production in hepatocytes through activation of AMPK. Moreover, low concentrations of metformin were found to activate AMPK by increasing the phosphorylation of AMPKα at Thr-172. However, the mechanism underlying the increase in AMPKα phosphorylation at Thr-172 and activation by metformin remains unknown. In the current study, we find that low concentrations of metformin promote the formation of the AMPK $\alpha\beta\gamma$ complex, resulting in an increase in net phosphorylation of the AMPK α catalytic subunit at Thr-172 by augmenting phosphorylation by LKB1 and antagonizing dephosphorylation by PP2C.

Metformin has been used for nearly a century (1) and is now the most widely prescribed oral anti-diabetic agent for the treatment of type 2 diabetes. The major effect of metformin is to reduce hepatic glucose production (2, 3), making metformin an ideal drug for controlling fasting hyperglycemia. However, its mechanism of action remains partly understood. A previous study reported that metformin increases net phosphorylation of the AMP-activated protein kinase (AMPK)[3] catalytic α subunit at Thr-172, with subsequent activation of AMPK activity in primary hepatocytes (4). Metformin has also been documented to inhibit complex 1 of the mitochondrial respiratory chain, which results in an increase in the AMP/ATP or ADP/ATP ratio, leading to AMPK activation through the binding of either AMP or ADP to AMPK (5, 6).

We recently showed that low metformin concentrations found in the portal vein suppress gluconeogenic gene expression and glucose production in cultured hepatocytes through AMPK (7). AMPK plays a key role in regulating metabolism and maintaining cellular energy homeostasis (8–10). AMPK, a phylogenetically conserved serine/threonine kinase, exists as a heterotrimeric complex, consisting of the catalytic α subunit and regulatory β and γ subunits (10). Each subunit has multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3), and AMPK subunits are encoded by seven different genes. Phosphorylation of α subunits at Thr-172 by upstream kinases such as the tumor-suppressor liver kinase B1 (LKB1) is critical for the activation of enzyme activity (11, 12). In contrast, phosphorylation of the α subunits at Ser-485/497 by PKA reduces α subunit phosphorylation at Thr-172 and decreases AMPK enzyme activity (7, 13–15). The AMPK β subunit has no catalytic activity, but the C-terminal domain of the β subunit interacts with the α and γ subunits, suggesting that it may function as a scaffold between the regulatory γ subunit and catalytic α subunit to form the AMPK heterotrimeric complex (10). The γ subunits have four cystathionine-β-synthase (CBS) domains, which bind adenine nucleotides. An increase in the AMP/ATP or ADP/ATP ratio results in a change in nucleotide binding to γ subunit and an allosteric change in AMPK (16). The allosteric change leads to an increase in net phosphorylation of α subunit at Thr-172 and AMPK activation either by augmenting phosphorylation by an upstream kinase or preventing dephosphorylation by a protein phosphatase (17).

Expanding on metformin-mediated activation of AMPK by increasing α subunit phosphorylation at Thr-172 (4), hepatic knock-out of LKB1, an upstream kinase for AMPKα phosphorylation, abolishes the effect of metformin to suppress hepatic glucose production (18). Activation of AMPK by metformin increases CBP phosphorylation at Ser-436 resulting in the disassembly of the CREB co-activator complex, inhibition of gluconeogenic gene expression and a reduction of glucose production (19). Numerous studies have confirmed that metformin inhibits mRNA or protein levels of the rate-limited gluconeogenic gene expression in the liver of mice and in primary hepatocytes (7, 20–24). Accordingly, in a P300G422S knock-in mouse model that bears a reconstituted metformin phosphor-

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[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed: Divisions of Metabolism and Endocrinology, 600 N. Wolfe St, Baltimore, MD 21287. Tel.: 410-502-5765; Fax: 410-502-5779; E-mail: heling@jhmi.edu.
[3] The abbreviations used are: AMPK, AMP-activated kinase; PP2C, protein phosphatase 2C; LKB1, liver kinase B1; CBS, cystathionine-β-synthase.

ylation site found in CBP, we found that this mouse model exhibits exaggerated hypoglycemia in a metformin tolerance test (25). Furthermore, phosphorylation of CRTC2 by activated AMPK leads to nuclear exclusion and degradation of CTRC2 in the cytoplasm (26). In the current study, we tested whether metformin mediated activation of AMPK by increasing net phosphorylation of the α subunit through promoting the formation of the AMPK heterotrimeric complex.

EXPERIMENTAL PROCEDURES

Plasmids and Adenoviruses—The FLAG-tagged AMPKβ1 and γ1 expression vectors were generated by subcloning the gene of mouse AMPKβ1 and γ1 into the p3XFLAG-CMV-7.1 expression vector (Sigma). For generation of adenoviral expression vectors, the mouse AMPKα1, β1, and γ1 genes were cloned into a pENTR2B vector (Invitrogen) and transferred into the pAd/CMV/V5-DEST vector (Invitrogen) by recombination to generate expression clones (27, 28).

Cell Cultures—Lipofectamine 2000 (Invitrogen) was used to transfect equal amounts of plasmids into mouse hepatoma cell lines (Hepa1–6) (27). After 48 h of transfection, cells were harvested, and cell lysates were subjected to immunoblot.

Animal Experiments—All animal protocols were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University. C57 mice were purchased from the Jackson Laboratory. Mice were fed on high-fat diet (60% calories), and metformin was given to the mice in their drinking water for 2 weeks (50 mg/kg body weight) (7).

Glucose Production Assays—Mouse primary hepatocytes were cultured in William's medium E supplemented with ITS (BD Biosciences) and dexamethasone. After 16 h of planting the primary hepatocytes, metformin was added into DMEM medium plus 10%FBS for 21 h, followed by 2 washes with PBS. After 3 h of serum starvation in DMEM supplemented with metformin, cells were washed twice with PBS, and the 1 ml glucose production medium was supplemented with metformin and glucagon. After 4 h incubation with glucose production medium, both the medium and cells were collected. The medium was used to determine glucose concentrations with the EnzyChrom Glucose Assay Kit (7, 27).

AMPK Activity Assay—The AMPK proteins were immunoprecipitated from cell lysates using anti-AMPKα antibody after incubation overnight at 4 °C. AMPK activity was determined by measuring the incorporation of ^{32}P into the synthetic SAMS peptide (abcam) as described previously (7).

Immunoprecipitation and Analysis of AMPK Phosphorylation and Dephosphorylation in in Vitro Assays—Cells were harvested in Cell Lysis Buffer (Cell Signaling) or RIPA buffer (Sigma). AMPKα1, β1, and γ1 were immunoprecipitated using β1/2 antibodies (Cell Signaling). FLAG-tagged AMPKα1, β1 and γ1 were immunoprecipitated from cell lysates using anti-FLAG M2 monoclonal antibody, following the procedure recommended by the manufacturer (Sigma). To assess the effect of metformin, phenformin, AMP, and ADP on the formation of the AMPK heterotrimeric complex, purified FLAG-tagged AMPKα1, and 2-fold of β1 and γ1 were incubated with different concentrations of metformin for 1–2 h at 4 °C, and then 1 μl of anti-AMPKα1 (abcam) was added. The reaction was incubated at 4 °C for 1h, followed by the addition of protein G beads (Active Motif) to pull down the AMPK heterotrimeric complex. To test the dose of LKB1 on the phosphorylation of AMPKα1, purified α1 was incubated with 80 μM metformin for 1h at 4 °C in the absence or presence of 2-fold of β1 and γ1 subunits, then different amounts of LKB1-STRAD-MO25 (Millipore) were added, and the reactions were incubated at room temperature for 30 min. In the time course experiment, 5-μl aliquots from each group were transferred at the indicated time points, and the kinase was inactivated immediately by heating at 95 °C for 5 min. In the dephosphorylation assays, purified α1 was first phosphorylated by LKB1-STRAD-MO25, then LKB1 was depleted by immunoprecipitation using LKB1 antibody. In the dephosphorylation assays, phosphorylated AMPKα1 was incubated with metformin for 1h at 4 °C in the presence or absence of 2-fold of γ1 and/or β1 subunits, then 75 ng of PP2C (Sigma) was added, and the reaction was incubated at 37 °C for 1 h.

Immunoblot—Immunoblots were conducted as previously described (27–29). Cell lysates were passed 15 times through a syringe needle or were sonicated for 15 s three times and immunoblotted to examine the target proteins with antibodies against AMPKα1, α2, β1, γ1 (abcam, Cell Signaling), and pAMPKα (Thr-172) (Cell Signaling) at the concentrations recommended by the manufacturers. Secondary antibodies were used at concentrations around 1:5000.

Statistical Analyses—Statistical significance was calculated with a Student's t test and ANOVA test. Significance was accepted at the level of $p < 0.05$.

RESULTS

Heterogeneous Expression of AMPK Subunits in Different Tissues—Our recent study showed that low metformin concentrations suppress glucose production through the activation of AMPK (7). Indeed, low metformin concentration (80 μM) activated AMPK and suppressed glucose production in primary hepatocytes (Fig. 1, *a* and *b*). To explore further the effect of AMPK in this pathway, we determined the protein levels of the AMPK subunits in different mouse tissues. As shown in Fig. 1*c*, the expression patterns of AMPK subunits are quite different. There are comparable amounts of α subunits in cellular lysates from the heart, kidney, and liver. However, the amounts of β2 and γ1/2/3 subunits are quite different in the cellular lysates from these tissues (Fig. 1*c*).

To estimate the cellular ratio of endogenous AMPK subunits, we generated adenoviral vectors to express FLAG-tagged AMPKα1, β1, and γ1, because they account for over 90% of AMPK activity in hepatocytes (30, 31). Each of the AMPKα1, β1 and γ1 subunits could be expressed both in primary hepatocytes and Hepa1–6 cells (Fig. 2*a*). Next, we expressed FLAG-tagged AMPK subunits at their endogenous levels (Fig. 2*b*), and then the pooled samples containing expressed FLAG-tagged AMPK subunits were immunoblotted with anti-FLAG specific antibody (Fig. 2*c*). In addition, FLAG-tagged AMPK subunits were expressed at their endogenous levels along with increasing amounts of their protein levels, and their relative expression levels were examined using anti-FLAG antibody (Fig. 2*d*). Our data suggest that β1 subunit is much more abundant than α1 and γ1 subunits in Hepa1–6 cells (Fig. 2, *c* and *d*). To more

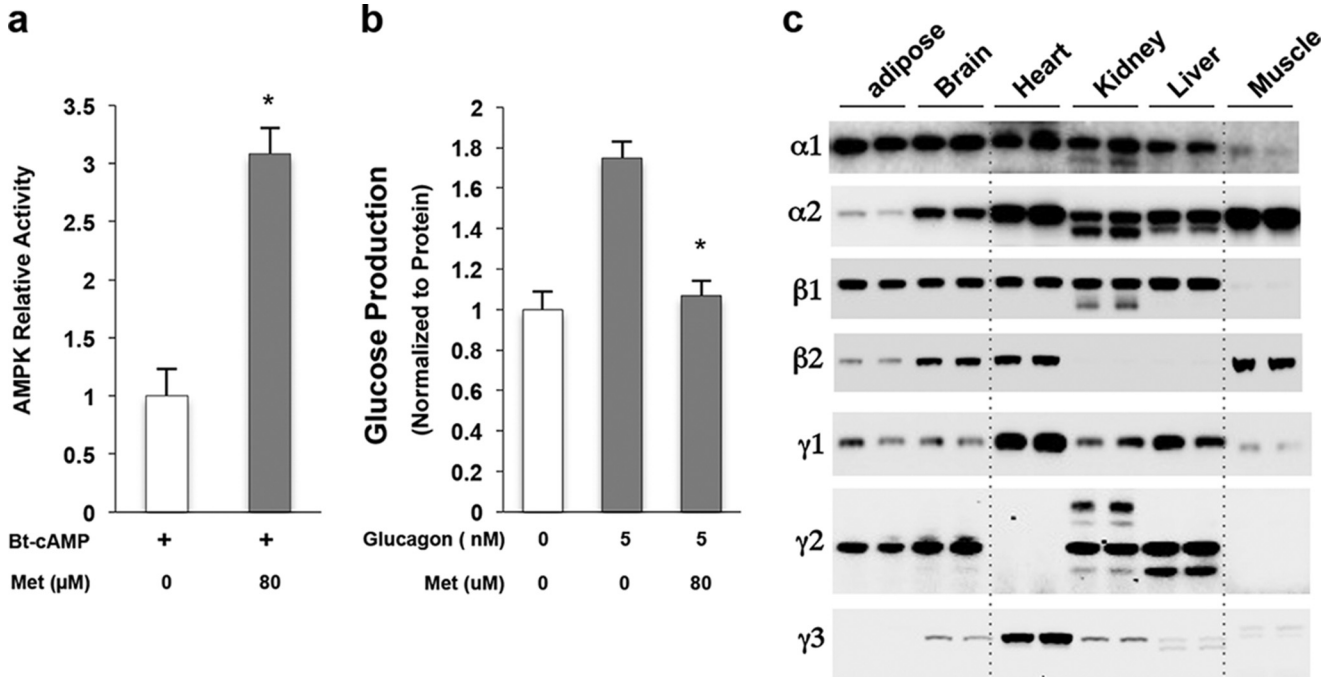

FIGURE 1. **A low metformin concentration activated AMPK and suppressed glucose production in mouse primary hepatocytes.** *a* and *b*, a low metformin concentration (80 μM) found in the portal vein after therapeutic dosage activated AMPK activity (*a*) and inhibited glucagon-stimulated glucose production in primary hepatocytes (*b*). Primary hepatocytes were treated as described under "Experimental Procedures." *c*, protein levels of AMPK subunits in different tissues (each lane represents a mouse sample (20 μg of cell lysate)). The *asterisk* (*) signifies that groups with same treatment are significantly different ($p < 0.05$).

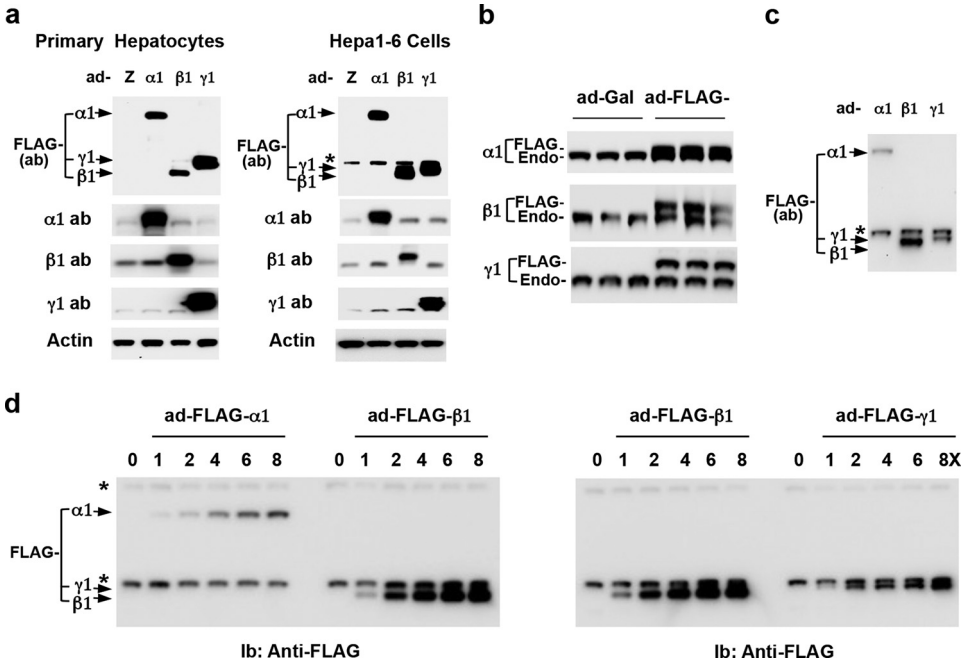

FIGURE 2. **Expression of AMPK α1, β1, and γ1 subunits in hepatocytes.** *a*, adenoviral FLAG-tagged AMPK subunits were added to primary hepatocytes (*left panel*) and Hepa1–6 (*right panel*). 48 h after incubation, cells were harvested, and cell lysates were subjected to immunoblot. *b*, FLAG-tagged AMPK subunits were expressed at their endogenous levels in Hepa1–6 cells. *c*, pooled samples (20 μg) of cell lysates from Hepa1–6 cells containing the expressed FLAG-tagged AMPKα1, β1 and γ1 in *b* were subjected to immunoblot using the anti-FLAG M2 antibody. *d*, FLAG-tagged AMPK subunits were expressed in Hepa1–6 cells with increasing amounts of their protein levels. Control denoted as "0" in each panel is the same cell lysate from Hepa1–6 cells without the addition of virus. In *lane 2* of each panel, FLAG-tagged AMPK subunits were expressed at their endogenous levels. Equal amounts (20 μg) of cell lysates were loaded and immunoblotted with anti-FLAG M2 antibody. *, nonspecific.

accurately assess their cellular protein ratio, we purified FLAG-tagged α1, β1 and γ1 subunits (Fig. 3*a*). The purified FLAG-tagged AMPK subunits were not associated with detectable endogenous AMPK subunits (Fig. 3*b*, *lower panel*). We determined the amounts of purified FLAG-tagged α1, β1, and γ1 subunits that were needed to match the corresponding protein levels in 20 μg of cellular lysates from Hepa1–6 cells (Fig. 3*c*,d). Then, the same amounts of purified FLAG-tagged α1, β1, and

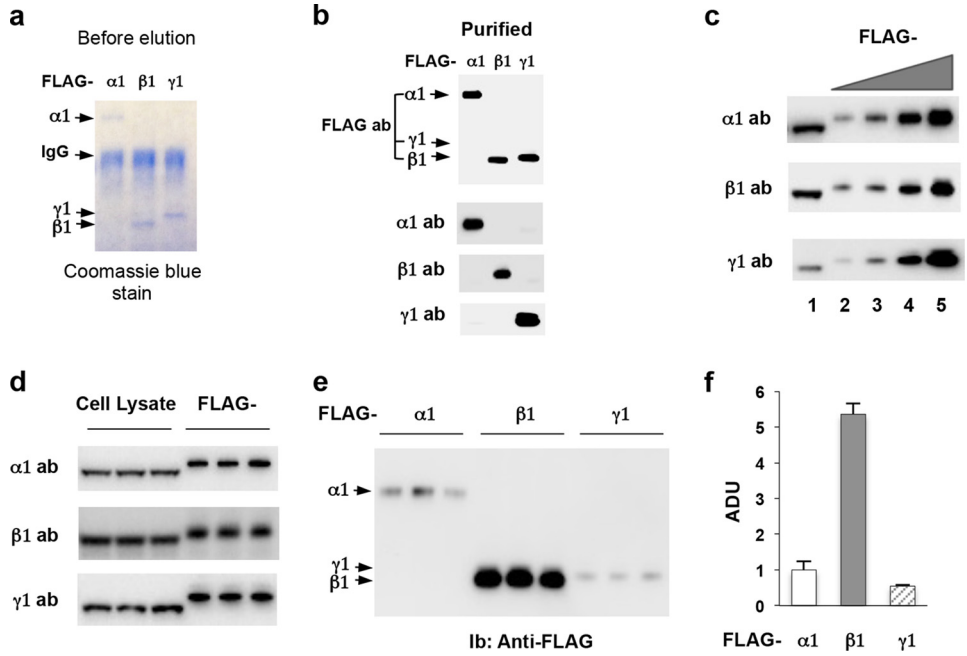

FIGURE 3. **Estimation of the cellular protein ratio of AMPK α1, β1, and γ1 subunits in Hepa1–6 cells.** *a*, cell lysates from primary hepatocytes containing the expressed FLAG-tagged AMPKα1, β1 and γ1 were incubated with anti-FLAG M2 magnetic beads and washed. Samples were taken before the final elution and were subjected to an SDS-PAGE gel and stained with Coomassie Blue. *b*, immunoblots of purified FLAG-tagged AMPKα1, β1, and γ1 with the indicated antibodies; equal amount of samples was loaded into each lane. *c*, to estimate the amounts of purified AMPK subunits that were needed to match their amounts in hepatocytes, 20 μg of cell lysates from Hepa1–6 cells (*lane 1*), and increasing amounts of purified FLAG-tagged AMPK subunits were loaded (*lanes 2–5*) and immunoblotted with the indicated antibodies. *d*, 20 μg of cell lysates from Hepa1–6 cells and the amounts of purified FLAG-tagged AMPK subunits that were needed to match the corresponding protein levels were loaded and immunoblotted with the indicated antibodies. *e*, amounts of purified FLAG-tagged AMPK subunits that matched their protein levels in cell lysate from Hepa 1–6 cells as in *d* were employed and immunoblotted with anti-FLAG M2 antibody. *f*, densitometric analysis of the protein levels as in *e*.

γ1 subunits that matched their endogenous protein levels in 20 μg of cellular lysates (Fig. 3*d*) were employed and immunoblotted with anti-FLAG antibody. As shown in Fig. 3, *e* and *f*, the α1β1γ1 subunits exist approximately in a 1:5:0.6 molar ratio in Hepa1–6 cells based on densitometry measurements.

Next, we assessed the ratio of α1β1γ1 subunits in the liver. First, we determined the amounts of purified FLAG-tagged α1, β1, and γ1 subunits that were needed to match the corresponding protein levels in 20 μg of mouse hepatic lysates (Fig. 4*a*). Subsequently, the same amounts of purified FLAG-tagged α1, β1, and γ1 subunits were used and immunoblotted with anti-FLAG antibody (Fig. 4*b*). In good agreement with the results from Hepa1–6 cells, we found that β1 subunit is much more abundantly than α1 and γ1 subunits in the liver. The α1β1γ1 subunits exist approximately in a 1:31:0.5 molar ratio (Fig. 4, *b* and *c*). To validate further these results, we used different commercial antibodies against α1, β1 subunits, and FLAG tag (Cell Signaling) and obtained similar results showing that there is ~32-fold higher β1 subunit protein levels than α1 subunit (data not shown).

Metformin Promoted the Formation of Endogenous AMPKαβγ Heterotrimeric Complex—The AMPK β subunit has no catalytic activity, but it interacts with both α and γ subunits, suggesting that it may function as a scaffold between the regulatory γ subunit and catalytic α subunit to form the AMPK heterotrimeric complex (10). Having seen the unequal endogenous expression of α1, β1, and γ1 protein levels (Figs. 3, *e* and *f*, and 4, *b* and *c*), we next asked whether metformin is able to affect the assembly of this AMPK heterotrimeric complex in hepatocytes. In untreated Hepa1–6 cells, β1 and γ1 bound together, and the α subunits did not associate with β1 and γ1 subunits (Fig. 5*a*, *left panel*). In contrast, in metformin-treated Hepa1–6 cells, immunoprecipitation with β1/2 antibodies depleted α subunits from the supernatant, and they were found in the pellet. Furthermore, phosphorylated α subunits (Thr-172) induced by metformin remained in a complex with β1 and γ1 subunits and was not found free in the supernatant (Fig. 5*a*, *right panel*). Metformin also increased the association of endogenous α with endogenous β and γ subunits in immunoprecipitation assays with antibodies against the β1/2 subunit (Fig. 5*b*). We observed increased association of endogenous α with endogenous β and γ subunits in Hepa1–6 cells treated with metformin regardless of the assay conditions (Fig. 5, *b* and *c*). In addition, metformin administration in mice fed a high-fat diet for 2 weeks led to increased α subunit phosphorylation at Thr-172 as well as the association of α subunit with β subunit in the liver (Fig. 5*d*).

To test further whether metformin increases AMPKαβγ heterotrimeric complex formation, we overexpressed a FLAG-tagged AMPKα1 subunit in hepatocytes. Anti-FLAG antibody was used to immunoprecipitate protein from cell lysates after metformin treatment. Metformin (250 μM) not only increased phosphorylation of FLAG-tagged AMPKα1 at Thr-172 but also increased the association of endogenous β1 and γ1 subunits with FLAG-tagged AMPKα1 subunit (Fig. 6*a*). Unbound endogenous β1 and γ1 subunits from control vehicle-treated cells remained in the supernatant in the immunoprecipitation assay. In agreement with the notion that the β1 subunit is more

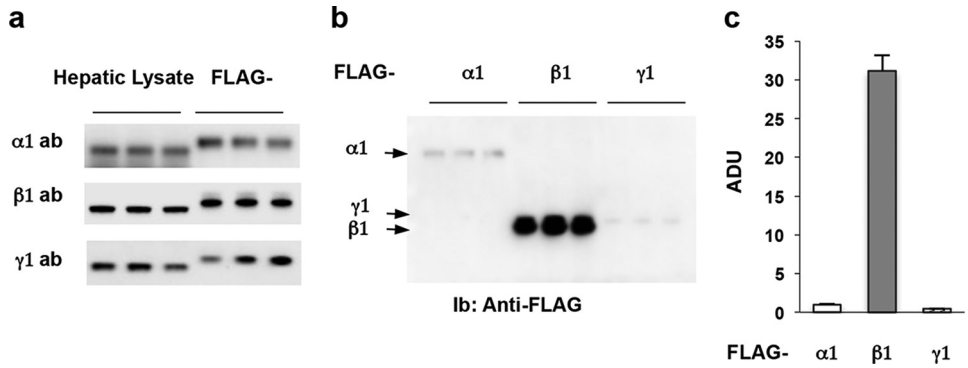

FIGURE 4. **Estimation of the cellular protein ratio of AMPK α1, β1, and γ1 subunits in the liver of mice.** *a*, 20 μg of cell lysates from mouse liver tissues and the amounts of purified FLAG-tagged AMPK subunits that were needed to match the corresponding protein levels were loaded and immunoblotted with the indicated antibodies. *b*, amounts of purified FLAG-tagged AMPK subunits that matched their protein levels in cell lysate from mouse liver tissues as in *a* were employed and immunoblotted with anti-FLAG M2 antibody. *c*, densitometric analysis of the protein levels as in *b*.

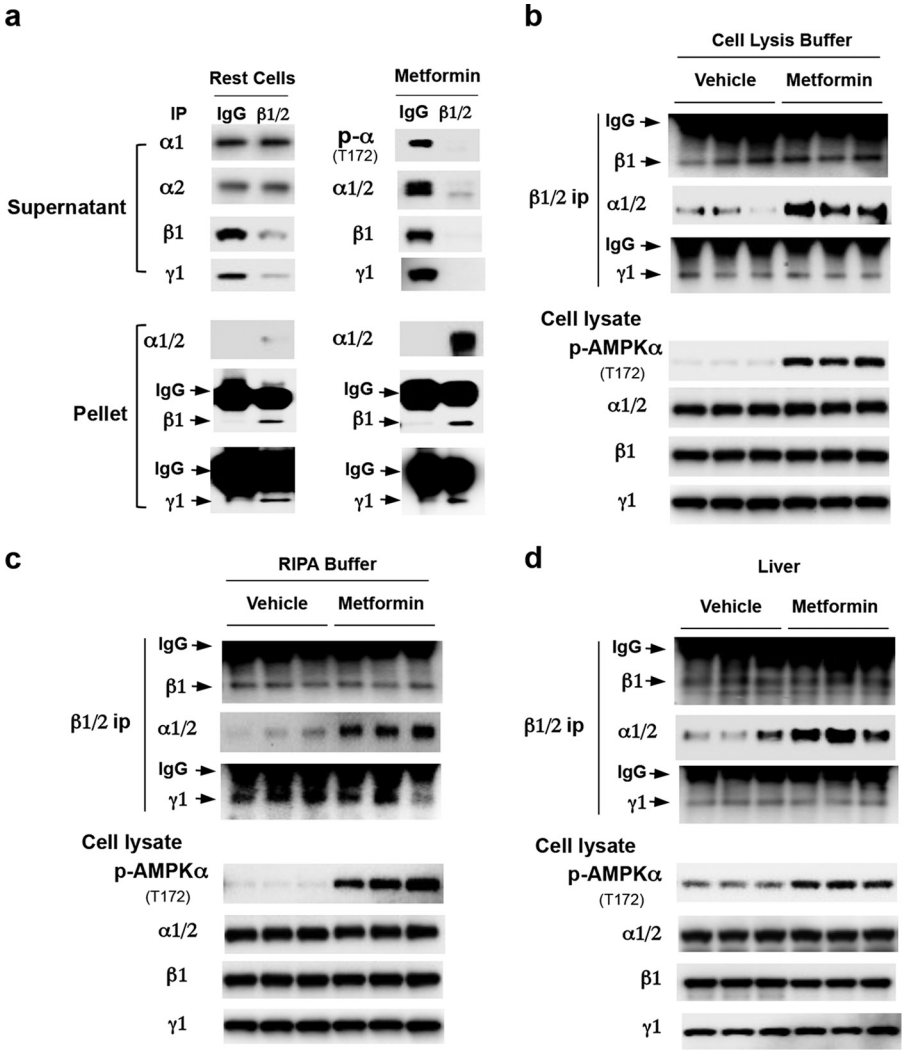

FIGURE 5. **Metformin promoted the formation of the AMPKαβγ heterotrimeric complex in hepatocytes.** *a*, cell lysates from untreated Hepa1–6 cells (*left panel*) and Hepa1–6 cells treated with metformin (0.5 mM, 5 h) (*right panel*) were incubated with normal IgG and antibodies against β1 and 2 together with protein G beads (16 h, 4 °C). Supernatant and magnetic beads (pellet) were collected separately. *b* and *c*, Hepa1–6 cells treated with metformin (0.5 mM. 5h) were harvested in Cell Lysis buffer (Cell Signaling) (*b*) or RIPA buffer (*c*) and incubated with antibodies against β1/2 (Cell Signaling). Immunoprecipitates were washed and immunoblotted with the indicated antibodies. *d*, metformin treatment (50 mg/kg, 2 weeks) increased the association of endogenous α subunit with endogenous β and γ subunits in the liver of mice fed a high-fat diet (each lane represents an individual mouse sample).

abundant than the α1 and γ1 subunits (Figs. 3, *e* and *f*, and 4, *b* and *c*), we only observed a small decrease in β1 in the supernatant after metformin treatment and immunoprecipitation (Fig. 6*a*). Moreover, metformin also increased the association of endogenous α1 with FLAG-tagged β1; unbound α1 was therefore reduced in the supernatant after immunoprecipitation

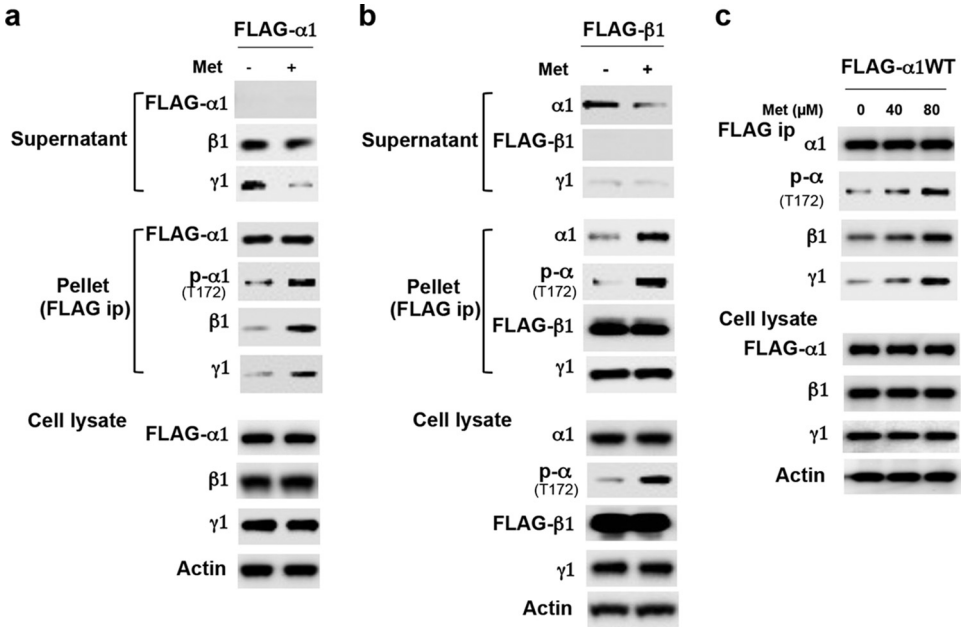

FIGURE 6. **Metformin promoted the association of FLAG-tagged AMPK subunits with endogenous partners to form the heterotrimeric complex in hepatocytes.** *a* and *b*, 48 h after the addition of FLAG-tagged AMPKα1 (*a*) or β1 (*b*) adenovirus, Hepa1–6 cells were subjected to serum starvation for 90 min, and then 0.25 mM metformin was added. Cells were harvested 5 h later. Cell lysates were incubated with anti-FLAG M2 magnetic beads for 16 h at 4 °C. The supernatant and magnetic beads (pellet) were collected separately, and the magnetic beads were washed twice. *c*, 48 h after the addition of adenoviral FLAG-tagged AMPKα1, the primary hepatocytes were treated with metformin at 40, 80 μM for 24 h.

(Fig. 6*b*). Lower concentrations of metformin had a similar effect on augmenting the association of endogenous β1 and γ1 subunits with FLAG-tagged α1 subunit in primary hepatocytes (Fig. 6*c*).

To probe further whether metformin affects the formation of the AMPK heterotrimeric complex directly or indirectly, we assembled the complex *in vitro* by combining 2-fold greater concentrations of purified β1 and γ1 subunits *versus* α1 subunit, treating the mixture with different concentrations of metformin for 1h and then immunoprecipitating α1 using an anti-α1-specific antibody. Low concentrations of metformin did indeed increase association of α1 subunit with β1 and γ1 subunits (Fig. 7, *a* and *b*), suggesting that metformin directly promotes the formation of the AMPK heterotrimeric complex. Consistent with the role of β subunit in bridging α and γ subunits, β subunit is able to bind to α subunit without the presence of γ subunit (Fig. 7*b*). Interestingly, another biguanide agent phenformin did not affect the formation of the AMPK heterotrimeric complex (Fig. 7*c*). In addition, AMP and ADP had no effect on the formation of AMPK heterotrimeric complex (Fig. 7*d*) and did not augment metformin-mediated AMPK heterotrimeric complex formation (Fig. 7*e*). However, metformin did not change AMPKα1 enzymatic activity in the absence or presence of β1 and γ1 subunits (Fig. 7, *f* and *g*). Importantly, assembly of the AMPK complex *in vitro* by metformin was concentration-dependent, reaching a maximal effect at ~100 μM; higher concentrations (0.5 and 1.0 mM) of metformin did not promote AMPK complex assembly.

Metformin Increased the Phosphorylation of AMPKα1 at Thr-172 by LKB1 in the Presence of β1 and γ1 Subunits—Activators of AMPK kinase, such as AMP, bind to the β or γ subunit and lead to an increase in net phosphorylation of Thr-172 on the α subunit. The increase in net phosphorylation has been attributed to either an increase in phosphorylation by an upstream kinase or a decrease in dephosphorylation by a protein phosphatase (32, 33). To assess the effect of the β and γ subunits on the α subunit phosphorylation at Thr-172, we overexpressed FLAG-tagged β1 and γ1 subunits in Hepa1–6 cells. Overexpression of FLAG-tagged β1 or γ1 subunit led to an increase in AMPKα phosphorylation at Thr-172, and overexpression of both plasmids further elevated net phosphorylation (Fig. 8*a*). Therefore, we tested *in vitro* whether both the β1 and γ1 subunits could affect the phosphorylation of α1 subunit by LKB1. As expected in this *in vitro* experiment, metformin alone was unable to stimulate phosphorylation of the α1 at Thr-172 even in the presence of the β1 and γ1 subunits (Fig. 8*b*). In contrast, after addition of LKB1-STRAD-MO25 (referred to as LKB1 kinase hereafter), phosphorylation of Thr-172 was observed and was much stronger in the presence of β1 and γ1 subunits (Fig. 8, *b* and *c*). In a time-course experiment, phosphorylation of the α1 subunit at Thr-172 was only seen in the presence of β1 and γ1 subunits (Fig. 8*d*). Higher concentrations of metformin did not affect AMPKα1 phosphorylation at Thr-172 by LKB1 kinase in the absence of the β1 and γ1 subunits (Fig. 8*c*). In comparison, Thr-172 phosphorylation of the α1 subunit by LKB1 kinase was maximal at 100 μM metformin in the presence of β1 and γ1 subunits and absent at higher concentrations of metformin (Fig. 8*e*). The phosphorylation of the α1 subunit at Thr-172 correlates well with the amount of β1 and γ1 subunits in the AMPKαβγ heterotrimeric complex (Figs. 7*a*, and 8, *e* and *f*). The γ1 subunit is required for metformin effect on Thr-172 phosphorylation of the α1 subunit by LKB1 kinase, because the metformin effect was lost when the γ1 subunit was absent in the reaction (Fig. 8*g*). We cannot exclude the possibility that high concentrations (0.5,

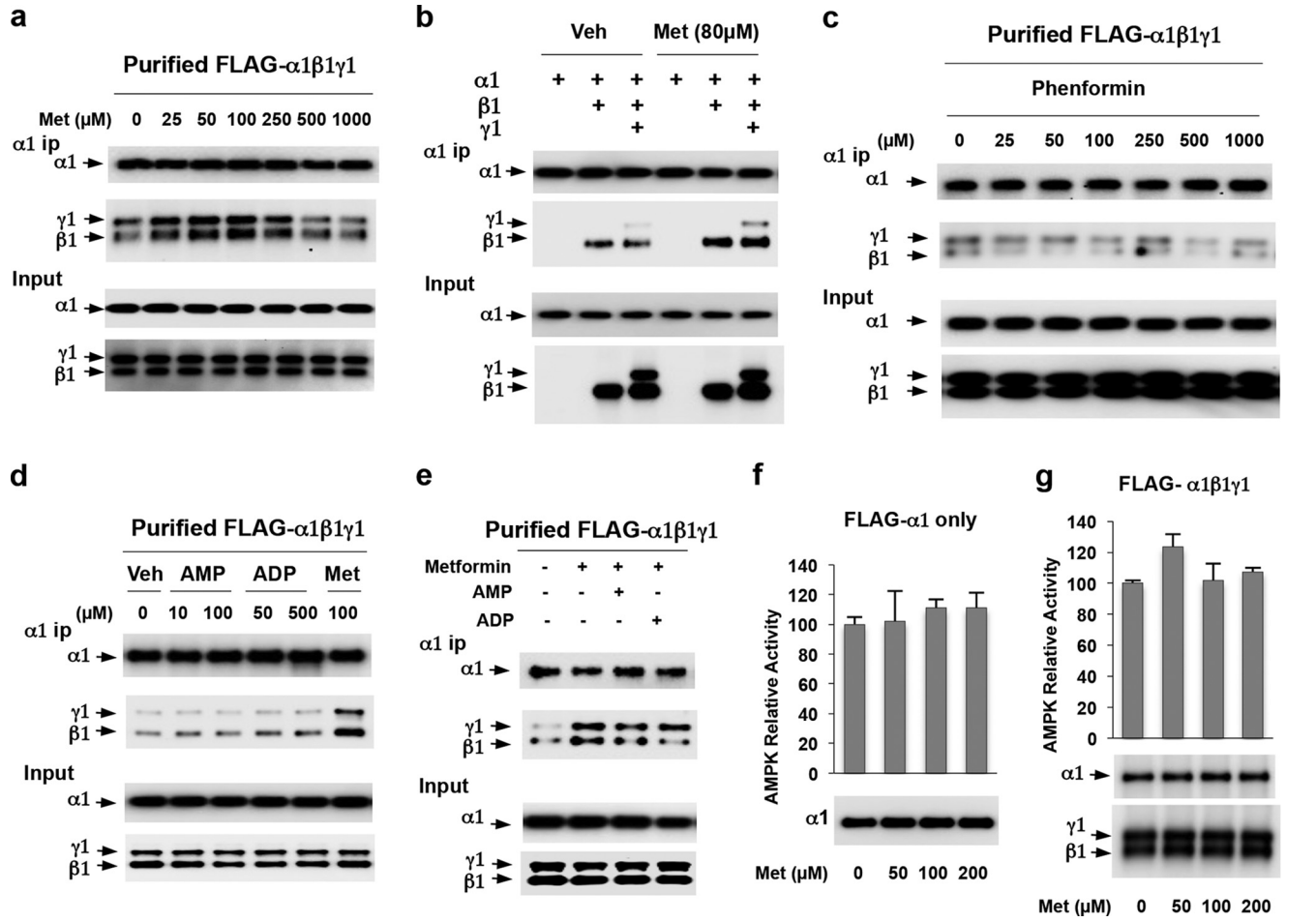

FIGURE 7. **Metformin promoted the formation of the AMPK$\alpha\beta\gamma$ heterotrimeric complex in *in vitro* assays.** *a* and *b*, purified FLAG-tagged AMPKα1, β1 and γ1 were combined and incubated with different concentrations of metformin, then immunoprecipitated with α1-specific antibody. The reaction was incubated at 4 °C for 1 h, followed by the addition of protein G beads to pull-down the AMPK heterotrimeric complex. Anti-FLAG antibody was used to examine the FLAG-tagged β1 and γ1 subunits. *c*, purified FLAG-tagged AMPKα1, β1, and γ1 were combined and incubated with different concentrations of phenformin, then immunoprecipitated with α1-specific antibody as in *a*. The reaction was incubated at 4 °C for 1 h, followed by the addition of protein G beads to pull-down the AMPK heterotrimeric complex. *d* and *e*, purified FLAG-tagged AMPKα1, β1, and γ1 were combined and incubated with different concentrations of AMP and ADP (*d*), and with 100 μM metformin together with 50 μM AMP or 200 μM ADP (*e*). The reaction was incubated at 4 °C for 1 h. *f* and *g*, incubation of metformin (24 h) did not directly affect AMPK enzymatic activity in the absence (*f*) or presence of β1 and γ1 subunits (*g*) ($n = 3$).

1 mM) of metformin might inhibit LKB1 activity directly, thereby decreasing phosphorylation of the α1 subunit at Thr-172 (Fig. 8*e*).

Metformin Decreased the Dephosphorylation of AMPKα1 at Thr-172 by Protein Phosphatase in the Presence of β1 and γ1 Subunits—To investigate further whether metformin has any effect on dephosphorylation of the α1 subunit at Thr-172, we used purified protein phosphatase 2C (PP2C), which has been shown to dephosphorylate the AMPKα1 subunit at Thr-172 in an *in vitro* assay (33, 34). In the absence of β1 and γ1 subunits, metformin had a minimal effect on preventing dephosphorylation of catalytic α1 subunit by PP2C (Fig. 9*a*). In contrast, at a low metformin concentration ($\leq$100 μM), dephosphorylation of the catalytic α1 subunit by PP2C was blocked in the presence of β1 and γ1 subunits (Fig. 9*b*). The γ1 subunit is dispensable for the block of dephosphorylation of the catalytic α1 subunit by PP2C due to the fact that low metformin concentrations prevented α1 subunit dephosphorylation at Thr-172 by PP2C in the absence of γ1 subunit (Fig. 9*c*).

DISCUSSION

Our recent study showed that low metformin concentrations found in the portal vein suppress glucose production through the activation of AMPK in hepatocytes (7). AMPK is an energy-sensing enzyme that is highly conserved and present in virtually all eukaryotes (10, 35). It is activated when cellular energy levels are low, triggering a switch from ATP-consuming anabolic pathways to ATP-producing catabolic pathways by stimulating glucose uptake and utilization and fatty acid oxidation together with reduction of hepatic glucose production. Originally, when AMPK was purified from the liver of rats, it was revealed that AMPK was associated with two other proteins, leading to the discovery of the β and γ subunits. Thus, functional AMPK is a heterotrimeric complex comprised of an α catalytic subunit and $\beta\gamma$ non-catalytic subunits (30, 31, 36, 37). It was suggested that AMPK α1β1γ1 or α2β1γ1 subunits exist approximately in a 1:1:1 molar ratio in the purified AMPK complex (30, 31). However, this ratio may not reflect the cellular protein ratio of AMPK subunits. Our results demonstrate, in fact, that the α1,

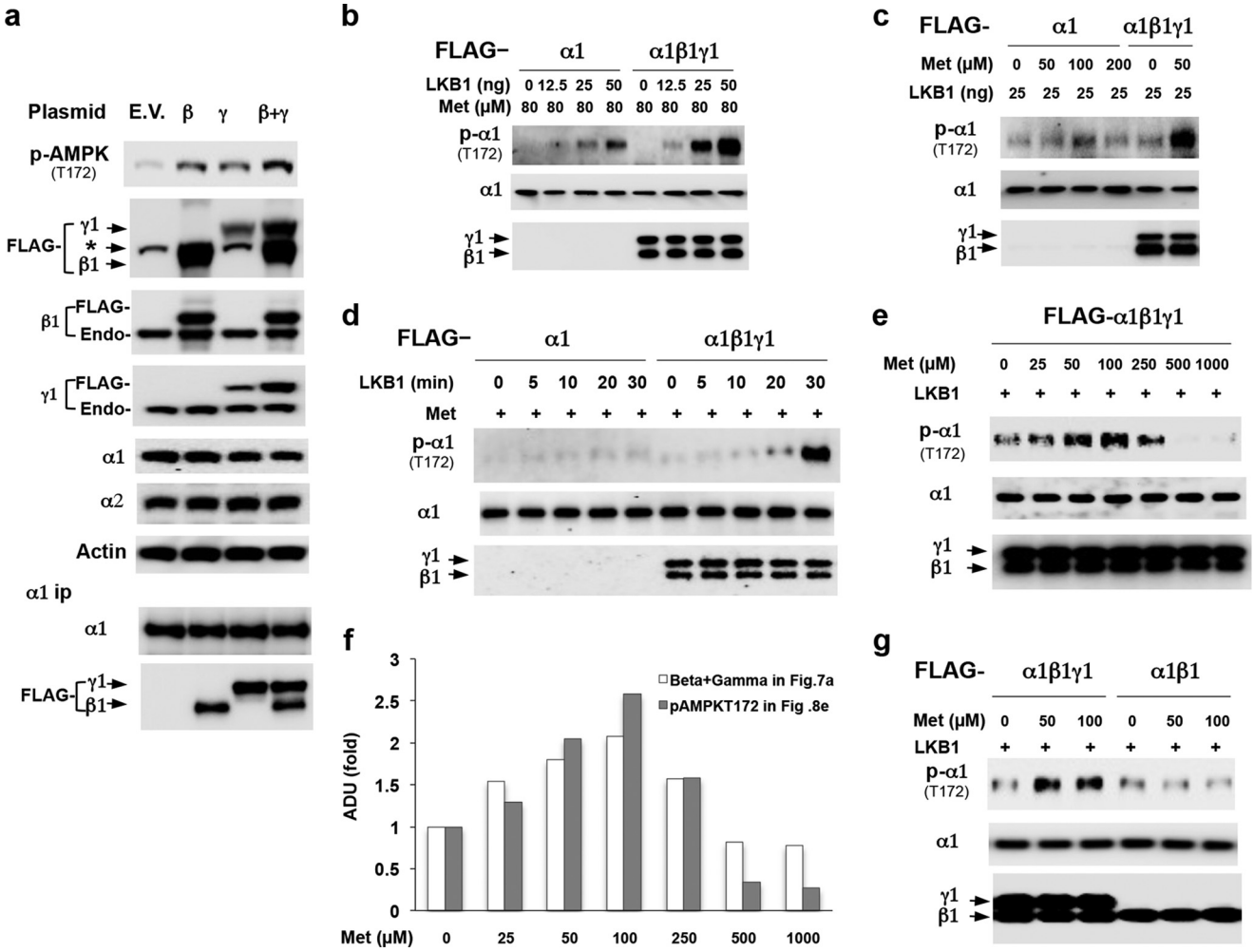

FIGURE 8. **Increased association of AMPK β and γ subunits with the α subunit led to increased phosphorylation of AMPK α at Thr-172 by LKB1.** *a*, 48 h after transfection with plasmids containing FLAG-tagged β1 and/or γ1, Hepa1–6 cells were harvested; cell lysates were subjected to immunoblots.*, nonspecific. *Endo*, endogenous. *b*, phosphorylation of purified α1 subunit by LKB1 kinase (30 min, room temperature) in the absence/presence of purified FLAG-tagged β1 and γ1 subunits in an *in vitro* assay. *c*, different metformin concentrations had minimal effects on the phosphorylation of purified α1 subunit by LKB1 in the absence of purified β1 and γ1 subunits in an *in vitro* assay. *d*, phosphorylation of purified α1 subunit by LKB1 only occurred in the presence of purified β1 and γ1 subunits in the time course *in vitro* assay, in which α1 was incubated with 80 μM metformin with or without β1 and γ1 subunits for 1 h at 4 °C before the addition of LKB1. *e*, purified α1, β1 and γ1 subunits were incubated with different amounts of metformin for 1 h at 4 °C before the addition of 25 ng of LKB1 in an *in vitro* assay. *f*, densitometric analysis of purified β1 and γ1 subunits associated with purified α1 subunit in Fig. 7*a* and the phosphorylation levels of purified α1 subunit at Thr-172 in *e*. *g*, purified α1, β1, and γ1 or α1 and β1 subunits were incubated with 50, 100 μM metformin for 1 h at 4 °C before the addition of 25 ng of LKB1 in an *in vitro* assay.

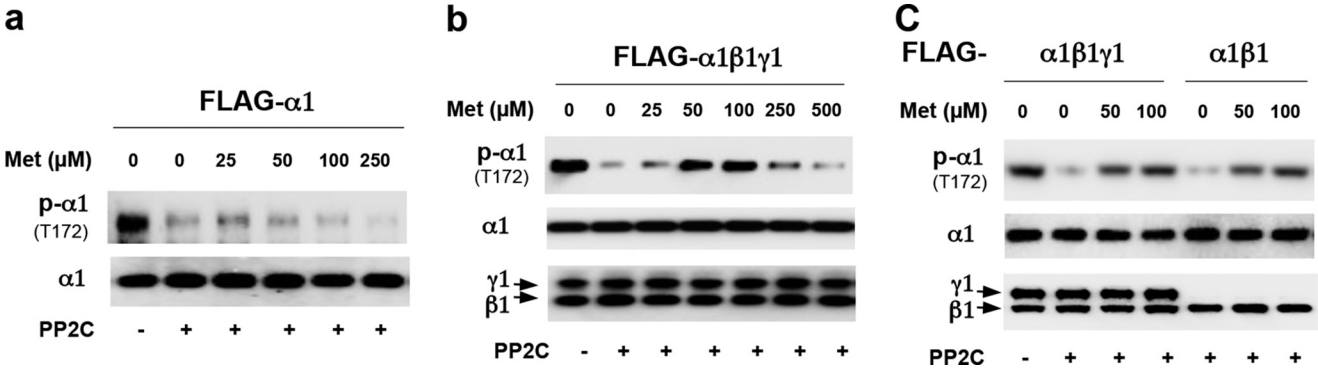

FIGURE 9. **Increased association of AMPK β and γ subunits with the α subunit prevented the dephosphorylation of AMPKα at Thr-172 by PP2C.** *a* and *b*, effect of different concentrations of metformin on the dephosphorylation of purified phosphorylated α1 subunit by 75 ng of PP2C in the absence (*a*) and presence (*b*) of purified β1 and γ1 subunits. The α1-specific antibody was used to examine the purified FLAG-tagged α1; anti-FLAG M2 antibody was used to examine the FLAG-tagged β1 and γ1 subunits. *c*, metformin antagonized the dephosphorylation of purified phosphorylated α1 subunit by 75 ng of PP2C in the presence of purified β1 and γ1 subunits or β1 subunit.

β1, and γ1 subunits do not exist in a 1:1:1 molar ratio in Hepa1–6 cells and liver tissues as in the purified AMPK complex. The β1 subunit is much more abundant than the α1 and γ1 subunits (Figs. 2, *c* and *d*; 3, e and *f*; and 4, *b* and *c*), suggesting that there might be unbound AMPK subunits in hepatocytes.

In this respect, we tested whether metformin has any effect on the formation of the AMPK heterotrimeric complex. Indeed, metformin promoted the formation of the AMPK heterotrimeric complex, which has been proved in three ways. First, metformin increased the association of endogenous α subunits with endogenous β and γ subunits (Fig. 5, *a* and *b*); similar results were also obtained using a different cell lysis buffer (Fig. 5, *b* and *c*). Importantly, metformin administration in mice fed a high-fat diet led to increased association of α subunits with endogenous β and γ subunits in the liver (Fig. 5*d*). Second, metformin augmented the association of β and γ subunits with FLAG-tagged α1 subunit in Hepa1–6 cells and primary hepatocytes, respectively (Fig. 6, *a* and *c*). Furthermore, metformin increased the association of the α1 subunit with FLAG-tagged β1 subunit (Fig. 6*b*). Phosphorylated α subunit (Thr-172) existed only in the complex (Figs. 5*a* and 6). Finally, metformin increased the association of FLAG-tagged α1 subunit with FLAG-tagged β and γ subunits in *in vitro* assays (Fig. 7, *a*, *b*, *d*, *e*), demonstrating that metformin binds directly to AMPK subunit to increase the formation of the AMPK heterotrimeric complex. Since β and γ subunits bind together in resting Hepa1–6 cells (Fig. 5*a*, *left panel*) and in hepatocytes without treatment (Figs. 5, *b*—*d* and 6), these data demonstrate that β and γ subunits appear to be preassembled before their association with α1 subunit. Interestingly, phenformin had no effect on the formation of the AMPK heterotrimeric complex (Fig. 7*c*), indicating that phenformin activates AMPK and suppresses hepatic glucose production through a potentially different mechanism.

Since the upstream AMPK kinase-LKB1 is in a constitutively active state (11), this indicates that metformin-mediated activation of AMPK occurs at AMPK complex. In light of this, metformin has been documented to activate AMPK by increasing α subunit phosphorylation at Thr-172 (4, 19, 32). We determined the importance of metformin-mediated formation of the AMPK heterotrimeric complex in the phosphorylation of α1 at Thr-172 by LKB1. Our results showed that low metformin concentrations drastically increased the phosphorylation of α1 subunit by LKB1. This occurred only in the presence of both β and γ subunits (compare Fig. 8, *c*, *e*, and *g*). In addition, we observed a pronounced increase in α1 phosphorylation at Thr-172 by LKB1 only in the presence of β and γ subunits during a time course experiment in which the same amounts of metformin and LKB1 were employed (Fig. 8*d*). Furthermore, metformin reduced the dephosphorylation of the α subunit at Thr-172 by protein phosphatase PP2C only in the presence of β and γ subunits, especially in the presence of β subunit (Fig. 9). It appears that γ subunit is not necessary for protecting against PP2C-mediated dephosphorylation of the α subunit at Thr-172 by metformin. Interestingly, high metformin concentrations (≥ 500 μM) did not promote the formation of the AMPK heterotrimeric complex (Fig. 7*a*) and did not increase α1 phosphorylation at Thr-172 by LKB1 even in the presence of β and γ subunits (Fig. 8*e*). This may be due to the fact that promiscuous binding of metformin to AMPK subunits at higher concentrations may interfere with the association of AMPK subunits because the -NH groups in metformin are able to form hydrogen bonds with amino acid residues in these subunits. Even though we found that low metformin concentrations suppress hepatic glucose production through the activation of AMPK, some metformin effects are reported to be AMPK-independent such as inhibition of mTORC1 activity (38).

In summary, we elucidate here the novel finding that metformin promotes the formation of the AMPK heterotrimeric complex, which leads to the activation of AMPK by increasing the net phosphorylation of AMPKα at Thr-172 through augmenting phosphorylation by LKB1 and antagonizing dephosphorylation by PP2C. Importantly, this occurred at low metformin concentrations (25–100 μM). We present data showing that metformin promotes the formation of the AMPK complex, but the binding site(s) of metformin on AMPK subunits still needs to be characterized.

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